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PATENT SPECIFICATION

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(54) A METHOD FOR THE PRODUCTION OF PROTEASE BY CULTIVATING BACTERIA

(71) We, GODO SHUSEI KABUSHIKA KAISHA, a Japanese Body Corporate, of No. 2-10, Ginza 6-chome, Chuo-ku, Tokyo, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for the production of an alkaline protease by cultivating bacteria.

It is well known to use bacteria of the genus *Bacillus subtilis* to produce an alkaline protease having activity at an optimum pH value within the high alkalinity zone (e.g. as described in "Nature", *170*, 802 (1952) by M. Ottessen and others, in "Journal of the Agricultural Chemical Society of Japan", *33*, 9 (1959) by Fukumoto and others, in "Agr. Biol. Chem.", *30*, 1261 (1966) by Fukumoto and others, or in "J. Biochem", *45*, 251 (1958) by Hagihara and others). Such an alkaline protease is widely used for food and industrial purposes but it should be noted that it is only produced by using strains of *Bacillus subtilis*.

Our investigations into the enzyme-producing activity of various kinds of bacteria isolated from the soil have now revealed that certain other bacteria have the strong power to produce protease. These bacteria were tested for their morphological, physiological and bacteriological properties by the procedures disclosed in Bergey's "Manual of Determinative Bacteriology", 7th edition, and were identified as belonging to *Bacillus licheniformis*.

A protease produced by *Bacillus licheniformis* was reported in "J. Biol. Chem.", *239*, 538 (1964) by R. W. Bernlohr, where it was stated that the protease was not inhibited by ethylene diamine tetra-acetic acid (E.D.T.A.), was similar to the protease produced by *Bacillus subtilis* in its thermal stability, and had activity over the wide range of pH values from 6.0 to 10.0 (but the optimum pH value was not made clear). An enzyme derived from *Bacillus licheniformis* was also described in "Biochem. Biophys. Acta.", *17*, 99 (1955) by N. Damodaran and others, which stated that the enzyme consisted of protease having the optimum pH value of 7.4 and peptidase. An enzyme derived from *Bacillus licheniformis* was reported in "Arch. Biochem. Biophys.", *114*, 145 (1966) by F. F. Hall and others, said to consist of two kinds of protease and one kind of peptidase, the protease in a casein-base culture medium having activity at optimum pH values ranging from 7 to 8.

The protease derived from Bacillus *licheniformis* which we have now found and identified is distinguished from the protease described in the above references by its properties, and particularly in the optimum pH value, which is an important factor in determining the activity and effect of the protease when it is used. The protease derived from *Bacillus licheniformis* which we have now found displays its activity at an optimum pH value within the high alkalinity zone from pH 10 to 10.5, even if casein, haemoglobin or albumin is used as the substrate, and is therefore obviously an alkaline protease.

Accordingly, this invention provides a new alkaline protease, produced by cultivating strains of *Bacillus licheniformis* found in the soil. This new alkaline protease can be used in many fields, including the manufacture of foodstuffs, the improvement of marine products, hides and foods, the textile industry, the manufacture of laundry product, and in other inductrial fields.

The invention consists in a process for producing an alkaline protease having activity over an optimum pH range from 10 to 10.5, which comprises cultivating strains of *Bacillus licheniformis* in a culture broth under aerobic conditions, and isolating the protease from the culture broth.

The culture broth may comprise any of the conventionally used sources of assimilable carbon and nitrogen, including glucose or starch as the carbon source and peptone or casein as the nitrogen source, as well as natural nutrients such as a yeast extract or a meat extract, and inorganic salts. An extract of defatted soybean cake, rice bran or corn

bran can also be used as the natural culture medium.

It is preferred to use a culture temperature of from 30 to 35°C at about pH 7.0 under aeration for 40 to 72 hours to obtain the maximum protease activity: for example, the maximum protease activity can be obtained at 35°C within 40 hours.

The alkaline protease accumulated in the culture broth may be recovered and purified by conventional techniques: for instance by successively carrying out the operations of de-bacterization, concentration, salting out, dialysis, de-colourization, column chromatography using an ion-exchange cellulose and an ion-exchange Sephadex (Registered Trade Mark) resin, and gel-filtration.

In the accompanying drawings, Figure 1 shows a chromatogram obtained by subjecting the culture broth to de-bacterization, salting out, dialysis, and de-colourization to obtain a purified alkaline protease and then subjecting the purified alkaline protease to column chromatography through carboxymethyl cellulose. In Figure 1, the curve (1) indicates the concentration of protein, the curve (2) indicates the protease activity and the straight line (3) indicates the concentration of sodium chloride. The protease activity is concentrated in the peak fraction (C) and also the activity of a small amount of protease can be observed in the fraction (D). The protease concentrated in the fraction (C) is the alkaline protease of the invention derived from *Bacillus licheniformis* and no fraction other than fractions (C) and (D) showed the activity of protease as may be seen in Figure 1. These facts show that the purification of the crude protease can easily be carried out and the advantageous effects of this invention can be achieved in producing the alkaline protease.

The characteristics of the alkaline protease produced in accordance with this invention are illustrated in Figures 2 to 5. Figure 2 shows the relationship between the activity of the alkaline protease and the pH value. As shown in Figure 2, the optimum pH value is found within the range of from 10 to 10.5 when casein is used as the substrate; and this optimum pH value is not altered even if haemoglobin or albumin is used as the substrate. Figure 3 shows the relationship between the activity of the alkaline protease and the temperature: curve (1) indicates the activity of the protease when the substrate contains 5×10^{-3} mol of calcium acetate, and curve (2) indicates the activity of the protease when the culture medium does not contain calcium acetate. Figure 3 shows that the optimum temperature is 60°C and the protease is de-activated at 80°C. The protease activity is remarkably increased by the presence of calcium acetate, but the optimum temperature is not substantially altered.

The activity of the alkaline protease was evaluated by the method described by Hagihara in "Methods for the Investigation of Enzymes", Vol 2., page 240 (Asakura Bookshop). The protease was mixed with a solution containing casein at pH 10.0 and 30°C, the casein was hydrolized to tyrosine and un-hydrolized casein was precipitated with "precipitating agent B" (a mixture of 0.11 mol of CCl_3COOH, 0.22 mol of CH_3COONa and 0.33 mol of CH_3COOH) and the light absorption of the filtrate was measured at 275 mμ. The "unit" in which the protease activity was measured is the amount of tyrosine (μ) which is produced in one minute.

Figure 4 shows the thermal stability of the alkaline protease: curve (1) shows that the protease is stable at temperatures up to 55°C when the reaction mixture contains 5×10^{-3} mol of calcium acetate, used at pH 10.0 for 15 minutes; and curve (2) shows that the protease is stable at temperatures up to 50°C, and its activity is rapidly reduced at temperatures above 55°C, when the culture medium contains no calcium acetate and is used under the same conditions as for curve (1).

Figure 5 shows the relationship between the protease activity and the pH value when the reaction mixture is held at 30°C for 22 hours: curve (1) shows the activity when the reaction mixture contains 5×10^{-3} mol of calcium acetate and the curve (2) shows the activity when the reaction mixture contains no calcium acetate. It is obvious from the curves (1) and (2) that the protease is stable at a pH from 5.0 to 11.0.

The activity of the alkaline protease of this invention is suppressed by heavy metal ions such as Hg and Cu ions, and particularly by oxidizers such as iodine or diisopropyl - fluorophosphate (DEP) and potato-inhibitor but not by reducing agents such as cysteine, chelating agents such as E.D.T.A., monoiodoacetic acid and SH reagent (a sulphhydryl enzyme inhibitor).

A strain of *Bacillus lichenformis* which is capable of producing an alkaline protease having activity over an optimum pH range of from 10.0 to 10.5 was deposited in the Fermentation Research Institute of the Agency of Industrial Science and Technology, Chiba City, Japan under the accession number FERM P-387 and in the American Type Culture Collection, Rockville, Maryland, U.S.A. under the accession number ATCC 21471. This strain, of which mutants may also be used, is hereinafter referred to as "FERM-P-387". The strain was characterised, as described in page 2, lines 1 to 10 of Bergey's Manual of

Determinative Bacteriology, 7th Edition (1957), as follows:

Vegetative rods : 0.6 to 0.8 by 1.5 to 3.0 microns, not in chains, no capsules or shadow-forms. Motile. Grampositive. On glucose nutrient agar the rods were the same as on nutrient agar and contained a few fat globules. Sometimes larger cells are observed.

Sporangia : Not definitely swollen, frequently resemble rods with bi-polar staining.

Spores : 0.6 to 0.9 by 1.0 to 1.5 microns, oval to cylindrical, central or paracentral, thin walled, many in 48 hours at 37°C.

Nutrient agar colonies : Rough surface, hairy outgrowths, spreading.

Nutrient agar slants : Growth abundant, rough, opaque adherent, spreading, matt surface, hairy outgrowths.

Nutrient broth : Clear with heavy wrinkled tough pellicle.

Gelatin stab : Rapid crateriform to stratiform liquefaction.

NaCl broth : Good growth in 5 to 8 per cent NaCl, no growth in 12 per cent NaCl.

Glucose nutrient agar slants : Growth heavy, rugose, extruded droplets and mucoid.

Tyrosine agar slants : Growth same as on nutrient agar.

Potato : Growth heavy, spreading, wrinkled, warty droplets extruded, pink, red to brown on submerged portion of potato.

Soybean agar slants : Growth softer and more abundant than on nutrient agar, brownish to reddish, droplets extruded.

Glucose nitrate agar slants : Growth slow, hairy outgrowth, later moderate to abundant growth.

Utilization of citrate : Positive.

Hydrolysis of starch : Positive.

Production of acetylmethylcarbinol : Positive (32°C)

pH of glucose broth : pH 5.6 at 7 days.

Fermentation tests : Acid without gas from arabinose, xylose, glucose, sucrose and mannitol (at 14 days)

Hydrolysis of casein : Positive, narrow zone of clearing.

Hydrolysis of gelatin : Positive, wide zone of hydrolysis.

Reduction of nitrate to nitrate : Positive.

Anaerobic production of gas from nitrate : Positive.

Anaerobic growth in glucose broth : Positive, pH 5.2 at 14 days, very small amount of gas produced.

Temperature for growth : Good growth 32 to 45°C.

The invention is further illustrated with reference to the following Examples.

Example 1

Bacillus licheniformis FERM - P - 387 was incubated in one litre of a liquid culture medium (pH = 7.2) containing 1% of glucose, 1% of peptone, 0.1% of yeast extract, 0.2% of sodium chloride, 0.2% of calcium chloride, 0.5% of potassium phosphate, 0.01% of magnesium sulphate, and 0.001% each of ferrous sulphate and manganese sulphate. The *Bacillus licheniformis* was cultured with shaking at 30°C for 72 hours. The resultant culture broth contained alkaline protease having an activity of 1,930 units per millilitre. The culture broth was de-bacterizated by using a centrifuge of filter, the filtrate was mixed with a 0.8 saturated solution of ammonium sulphate and the mixture was subjected to salting out and dialysis. The dialyzed solution was passed through a column packed with Duolite A-2 resin (anion-exchange resin) to remove colouring matter. The decoloured solution was subjected to column chromatography using carboxymethyl cellulose and DEAE-Sephadex, and then the crude protease was separated by gel-filtration using Sephadex-75. "DUOLITE" and "SEPHADEX" are Registered Trade Marks. A purified protease was obtained by precipitation with acetone, and dried. The dried protease weighed 47 milligrams and had an activity of 4,110 units per milligram.

Example 2

Bacillus licheniformis FERM-P-387 was incubated in one litre of a liquid culture medium (pH = 7.2) containing an extract which was produced by extracting defatted soybean cake with a 4% alkaline solution and 1% of soluble starch; and the *Bacillus licheniformis* was cultured in a jar-fermenter at 35°C for 40 hours under aeration and agitation. The resultant culture broth contained alkaline protease having an activity of 4,400 units per millilitre. This culture broth was treated in the same manner as in Example 1, producing 1.07 grams of purified dried protease having an activity of 4,110 units per milligram.

WHAT WE CLAIM IS:—

1. A process for the production of an alkaline protease having activity over an optimum pH range from 10 to 10.5, which comprises cultivating strains of *Bacillus licheniformis* in a culture broth under aerobic conditions, and isolating the protease from the culture broth.

2. A process according to claim 1, in which the cultivation is carried out at a pH of about 7.0.

3. A process according to claim 1 or claim 2, in which the cultivation is carried out at a temperature from 30 to 35°C.

4. A process according to any preceding claim, in which the cultivation is continued for a period of time from 40 to 72 hours.

5. A process according to any one of the preceding claims, in which said strain is FERM - P - 387 or a mutant thereof.

6. A process according to claim 1, substantially as herein described in either of the foregoing Examples.

7. An alkaline protease having activity over an optimum pH range from 10 to 10.5 when produced by the process of any preceding claim.

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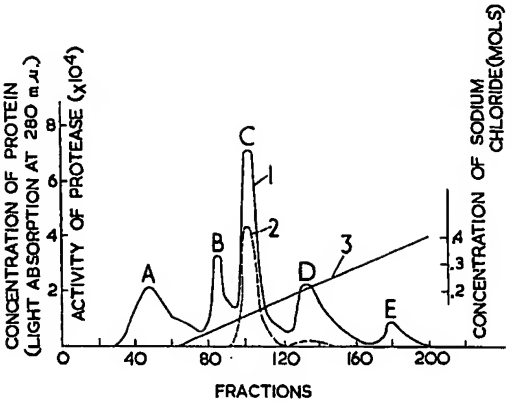

FIG.1.

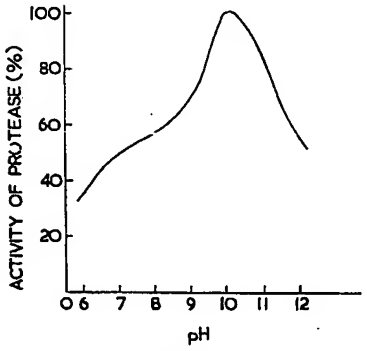

FIG.2.

1263765 COMPLETE SPECIFICATION
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Sheet 2

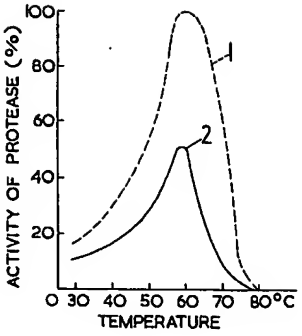
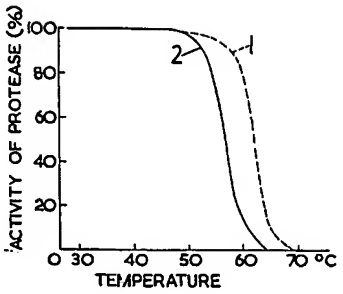


FIG.3.

FIG.4.



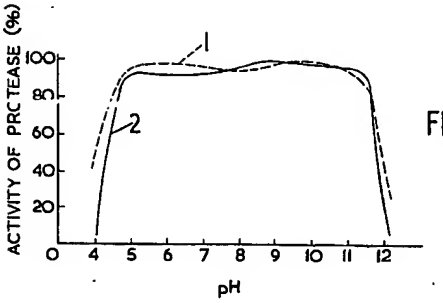


FIG.5.